

REMARKS

Reconsideration is requested.

Claims 1-34, 36, 39-42 and 45 have been canceled, without prejudice.

Claim 35 has been amended to specify that the rodent is a mouse having a mutated LAT gene encoding a mutant LAT protein, wherein the sequence of said mutant LAT protein differs from a mouse wild type sequence by a single mutation of a tyrosine located at position 136 of the wild-type mouse LAT protein sequence, which mutation is not a composite mutation of the tyrosine residues at positions 175, 195, and 235 of said wild-type mouse LAT protein sequence, said tyrosine located at position 136 being replaced by a residue preventing the association with the SH2 domain of proteins, said mouse being homozygous for the mutated LAT gene or being a carrier of a null allele of the LAT gene, and said mutant LAT protein leading to an exaggerated TH2 cell differentiation.

Revisions to claims 35 and 57 find support, for example, in now-cancelled claim 42, and at page 8, lines 8-13 of the specification.

Claims 37, 43, 44, 46, 47 and 64 have also been amended to specify, in accordance with claim 35, that the rodent is a mouse.

These amendments are made without prejudice or disclaimer and solely in order to facilitate reconsideration of this application. In particular, applicant reserves his right to file a divisional application at a later stage, and the present amendment shall not be considered as an admission of the objection or as a waiver of any subject matter.

No new matter has been added. The claim amendments are not believed to raise new issues requiring further search and/or consideration. Entry of the present Amendment is requested.

The objection to claim 47 is obviated by the above amendments. Entry of the present Amendment and withdrawal of the objection to claim 47 is requested.

To the extent not obviated by the above amendments, the Section 112, first paragraph "enablement" and "written description", rejections of claims 35, 37, 41-44, 46, 47 and 57-61 are traversed. Reconsideration and withdrawal of the rejections are requested in view of the following comments.

Claim 35 and dependent claims above specify that: the non human animal is a mouse, and that the mutation is a single mutation of a tyrosine located at position 136 of the wild-type mouse LAT protein sequence, which mutation is not a composite mutation of the tyrosine residues at positions 175, 195, and 235 of said wild-type mouse LAT protein sequence, said tyrosine located at position 136 being replaced by a residue preventing the association with the SH2 domain of proteins.

The applicants believe one of ordinary skill in the art will be able to practice the claimed invention without undue experimentation and that one of ordinary skill will appreciate from the present specification that the applicants were in possession of the claimed invention at the time the application was filed.

The specification provides three particular examples of mutation which affect the tyrosine located at position 136 of the wild-type mouse LAT protein sequence and are able to prevent the association of LAT with the SH2 domain of proteins. These particular examples of mutation consist in the replacement of the tyrosine located at position 136

of the wild-type mouse LAT protein sequence by a phenylalanine (Y-F), an aspartic acid (Y-D) or a glutamic acid (Y-E) (see page 6, lines 11-17 of the specification).

Applicants believe that the examples of mutations provided in the specification are easily used by the person of ordinary skill in the art to predict the phenotype of a mouse comprising a distinct mutation of the tyrosine located at position 136, preventing the association with the SH2 domain of proteins, without undue experimentation.

Applicants further provide herewith a Declaration from an inventor of the present invention (Mr. Bernard Malissen). Results provided in the Declaration demonstrate that a mouse carrying a null allele of the LAT gene also has a phenotype of increased TH2 cells.

Entry of the present Amendment and withdrawal of the Section 112, first paragraph, rejections are requested.

Entry of the above amendments will, in the applicants view, obviate the Section 112, second paragraph, rejection of claims 35, 37, 41-44, 47 and 58-61. Entry of the Amendment and withdrawal of the Section 112, second paragraph, rejection are requested.

To the extent not obviated by the above amendments, the Section 102 rejection of claims 47 and 57-61 over Sommers (Journal of Experimental Medicine, 194(2), 135-142), is traversed. Entry of the present Amendment and withdrawal of the Section 102 rejection are requested in view of the following distinguishing comments.

Sommers et al. is not believed by the applicants to describe or suggest to prepare, a mutated mouse gene according to the invention, coding for a mutant LAT protein, the sequence of which differs from a wild-type mouse LAT sequence by a single

mutation of a tyrosine located at position 136 of the wild-type mouse LAT protein sequence, which mutation is not a composite mutation of the tyrosine residues at positions 175, 195, and 235 of said wild-type mouse LAT protein sequence, said tyrosine located at position 136 being replaced by a residue preventing the association with the SH2 domain of proteins, or a cell comprising such a gene.

As appears to be acknowledged by the Examiner, Sommers et al. do not describe nor suggest the phenotype (of exaggerated TH2 cell differentiation) that should be observed in a mouse comprising such a mutant LAT protein. On the contrary, Sommers et al. are believed by the applicants to teach away from the invention by stating that "*no mature T cells were present*" in the studied LAT "knock-in" mutant mice having a mutation of the four distal tyrosines, at positions 136, 175, 195 and 235.

Withdrawal of the Section 102 rejection is requested.

The claims are submitted to be in condition for allowance. Rejoinder and allowance of any claim defining a method of making and/or using a product defined by an allowable claim, at an appropriate time, are requested. Entry of the present Amendment and allowance of the application are requested. The Examiner is requested to contact the undersigned in the event anything further is required in this regard.

MALISSEN et al
Appl. No. 10/502,332
June 20, 2007
Amendment After Final Rejection

Respectfully submitted,

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re- application of Malissen et al

Serial No. 10/502,332

Group art Unit: 1632

Examiner: Ton, THAIAN N

Filed 07/23/2004

For: "Mutated gene coding for a LAT protein and the biological applications thereof."

DECLARATION UNDER RULE 132

Hon. Commissioner of Patents and Trademarks
WASHINGTON D.C. 20231

Sir:

I, Bernard Malissen, residing at 11 Avenue de la Pinède, 13009 MARSEILLE, France,

Declare and Say:

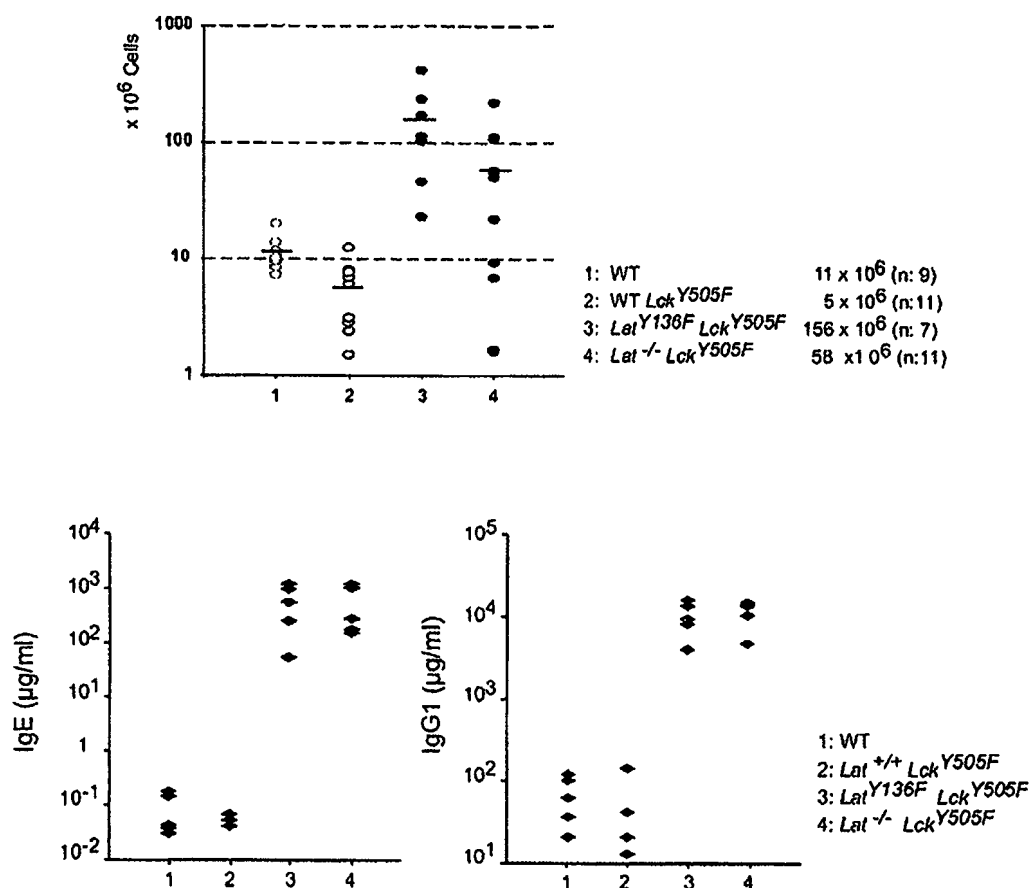
I am a citizen of France.

I am Doctor in Science (PhD in 1982) and permanent researcher ("Directeur de Recherche") at the INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM).

I am an inventor of the present patent application (Serial No. 10/502,332).

I read the Office Action from the USPTO, dated 02/20/2007, and I have the following comments:

We have generated additional data emphasizing our claims and illustrated by the following Figures:



The first figure (upper panel) shows that CD4⁺ αβ T cells with a memory phenotype expand over time in the spleen and lymph nodes of mice homozygous for a null allele of the *Lat* gene (*Lat*^{-/-}) and expressing a gain-of-function form of the Lck tyrosine kinase (*Lck*^{Y505F}). Splens of 6- to 9-week-old mice were analyzed for CD4⁺ T cell content. Results for individual splens were plotted on a logarithmic scale (Key, genotype and number (n) of mice analyzed) ---- represents mean of each distribution.

The second and third figures (bottom panels) show that the expanding CD4⁺ αβ T cells develop a Th2 effector phenotype as documented by serum levels of IgG1 and IgE antibodies samples from 6 to 9 week-old mice. The concentrations of IgG1 and IgE in individual mice are plotted on a logarithmic scale (Key, genotype of mice).

These data demonstrate that a Th2 pathology similar to that appearing in *Lat*^{Y136F} mice can be observed in the absence of LAT.

The Src-family protein tyrosine kinase Lck constitutes an important component of the pre-TCR and TCR signalling cassettes. When introduced into thymi of mice that are deprived of functional pre-TCR and thus contain T cells that are blocked at an early stage of development (called the "DN cell stage"), a gain-of-function form of the Lck tyrosine kinase, in which the inhibitory tyrosine residue found at position 505 is mutated to phenylalanine (*Lck*^{Y505F}), can

functionally substitute for the missing pre-TCR and rescue T cell development (Mombaerts et al. Immunity 1: 261, Aug 1995).

We observed that breeding the Lck^{Y505F} mutant allele onto a LAT-deficient background (i.e. mice homozygous for a null allele of the *Lat* gene, $Lat^{-/-}$) relieved the developmental block observed in *Lat*-deficient thymi at the DN cell stage. The few $CD4^{+}$ T cells that reached the periphery of $Lat^{-/-}$, Lck^{Y505F} mice spontaneously deployed a Th2 effector program and embarked into a polyclonal lymphoproliferative disorder identical to the one that unfold in mice expressing LAT Y136F molecules (see above Figures). Therefore, when T cell precursors harbouring null allele of *Lat*, are allowed to develop up to the $CD4^{+}$ stage, through a pulse of Lck^{Y505F} inducer that stops when those cells leave the thymus, they also undergo the same lymphoproliferative disorder and differentiation into Th2 effectors as Lat^{Y136F} $CD4^{+}$ T cells do.

The undersigned Declarant declares further that all statements made herein of this own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this day of June 12, 2007



Bernard MALISSEN

An Activated *lck* Transgene Promotes Thymocyte Development in *RAG-1* Mutant Mice

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Summary

Expression of the T cell receptor β (TCR β) chain is necessary for the transition from the CD4⁺CD8⁺ stage in the major $\alpha\beta$ thymocyte lineage. The protein tyrosine kinase p56^{lck} has been implicated in the regulation of early thymocyte differentiation and of allelic exclusion at the TCR β locus. Using mice overexpressing an activated *lck* transgene and mice with a disruption of the *lck* gene, we demonstrate that p56^{lck} participates in a pathway that regulates the expansion of the pool of CD4⁺CD8⁺ thymocytes to wild-type levels. In addition, p56^{lck} may be involved in the down-regulation of the putative pre-TCR on CD4⁺CD8⁺ thymocytes.

Introduction

The main pathway of $\alpha\beta$ thymocyte differentiation consists of a series of stages that can be defined by expression of various surface markers (von Boehmer, 1988). The major stages are characterized by the presence or absence of the coreceptors CD4 and CD8. Immature thymocytes progress from the CD4⁺CD8⁺ (double negative, or DN) to the CD4⁺CD8⁺ (double positive, or DP) stage. At the DP stage, $\alpha\beta$ thymocytes interact through their heterodimeric $\alpha\beta$ TCR with class I or class II major histocompatibility complex molecules expressed on thymic stromal cells. Subsequent TCR-driven positive and negative selection mechanisms permit the export of CD4⁺CD8⁺ or CD4⁺CD8⁺ single positive T cells to the periphery. The TCR of $\alpha\beta$ T cells is a clonally variable heterodimer of α and β chains

(Davis and Bjorkman, 1988). TCR α and TCR β genes, like immunoglobulin genes, are assembled from variable (V), diversity (D), and joining (J) gene segments through the process of V(D)J recombination (Tonegawa, 1983), which is dependent on the recombination activating gene 1 (*RAG-1*) and *RAG-2* (Schatz et al., 1989; Oettinger et al., 1990; Mombaerts et al., 1992a; Shinkai et al., 1992). Analysis of mice with mutations in *RAG-1* or *RAG-2*, or in TCR α , TCR β or TCR δ genes, revealed that TCR β gene rearrangement or expression is an important regulator of the progression of DN thymocytes to the DP stage and the expansion of the pool of DP cells (Mombaerts et al., 1991, 1992a, 1992b; Philpott et al., 1992; Shinkai et al., 1992, 1993; Mombaerts and Tonegawa, 1994).

The DN TCR-negative thymocyte population can be subdivided into four populations, based on surface expression of CD44 (phagocytic glycoprotein-1) and CD25 (IL-2-receptor- α chain) (Godfrey and Zlotnik, 1993). The pathway of differentiation has been defined as follows: CD44⁺CD25⁺ \rightarrow CD44⁺CD25⁺ \rightarrow CD44⁺CD25⁺ (Godfrey et al., 1993). In wild-type mice, TCR β gene rearrangements occur at the CD44⁺CD25⁺ stage, and thymocyte development is blocked at this stage in *RAG-1* mutant mice or in TCR β \times δ double mutant mice (Godfrey et al., 1994). Surface expression of a pre-TCR, containing TCR β without TCR α , on immature thymocytes may be dependent on a putative surrogate TCR α chain, gp33 (Groettrup et al., 1993).

The nonreceptor protein tyrosine kinase p56^{lck} is expressed in thymocytes from the time that hematopoietic progenitors first colonize the thymic anlage, and *lck* transcripts continue to be present throughout thymocyte development (reviewed by Perlmutter et al., 1993). This kinase is also involved in signaling in mature T cells, in part through its interactions with the cytoplasmic tails of CD4 and CD8. Recently, several studies have implicated p56^{lck} in signal transduction during TCR β chain-dependent early thymocyte differentiation (reviewed by Owen, 1993; Anderson et al., 1994). First, mice carrying a targeted mutation in the *lck* gene manifest thymic abnormalities analogous to those seen in TCR β mutant mice, although the reduction in the numbers of DP thymocytes is somewhat less (Molina et al., 1992). Second, in mice expressing a dominant negative *lck* transgene, few DP thymocytes exist, and in the transgenic lines expressing the highest levels of this catalytically inactive form of p56^{lck}, only DN thymocytes are observed (Levin et al., 1993a). TCR β loci but not TCR α genes were extensively rearranged in these thymocytes (Levin et al., 1993a). Expression of a functionally rearranged TCR β transgene was unable either to induce differentiation beyond the block or to exert allelic exclusion at the TCR β locus (Anderson et al., 1993). Third, in transgenic mice overexpressing either wild-type or constitutively active p56^{lck}, DP thymocytes lacking V-D-J TCR β gene rearrangements but expressing V-J TCR α transcripts were generated in near-normal numbers, suggesting that p56^{lck} can deliver a signal analo-

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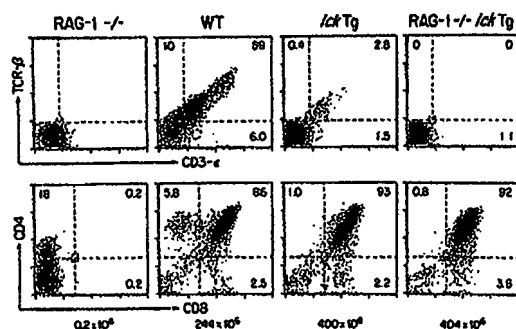


Figure 1. Flow Cytometric Analysis of Thymocytes

(Top) Staining with CD3ε-FITC and TCRβ-PE.

(Bottom) Staining with CD8-FITC and CD4-PE. Four littermates of approximately 3 weeks of age are shown. The transgene was pLGC4, line 7120. The numbers of total thymocytes are indicated at the bottom of each type of mouse.

gous to that which follows TCRβ expression (Abraham et al., 1992; Anderson et al., 1992). Transgenes encoding a form of p56^{lck} that is unable to bind to either CD4 or CD8 exerted similar effects on thymocyte development and TCR gene rearrangements, demonstrating that the signal transmitted via p56^{lck} acts independently of coreceptor expression (Levin et al., 1993b). Taken together, these studies strongly suggest that p56^{lck} may transduce, at least in part, the signal that emanates from expression of a rearranged TCRβ chain, a signal that induces both differentiation beyond the CD44⁺CD25⁺ stage and clonal expansion.

The data reported in this paper reinforce the view that p56^{lck} participates in a pathway required for TCRβ chain-mediated differentiation beyond the DN stage, and is probably essential for subsequent clonal expansion of DP

thymocytes. p56^{lck} also appears to be required to down-regulate surface expression of the pre-TCR that is postulated to direct maturation to the DP stage. These signaling properties of p56^{lck} are independent of its ability to interact with CD4 or CD8.

Results

lck Transgenic *RAG-1* Mutant Mice

To find out whether p56^{lck} can substitute for the effect of TCRβ on the maturation of DN thymocytes, we crossed several lines of transgenic mice overexpressing an activated form of p56^{lck} with *RAG-1* mutant mice. The *lck* transgenes contain a tyrosine to phenylalanine mutation at codon 505, which yields protein with approximately 7-fold greater catalytic activity. We employed both transgenes encoding activated p56^{lck} capable of binding to CD4 and CD8 (construct pLGF) (Abraham et al., 1992), or a transgene with additional cysteine to alanine substitutions at positions 20 and 23, which together render the protein unable to bind to CD4 and CD8 (construct pLGC4) (Levin et al., 1993b).

When the *lck* transgenes were introduced into the *RAG-1* mutant background, DP thymocytes appeared in large numbers. More than 90% of the thymocytes were DP in these mice (Figure 1). The total number of thymocytes in *lck* transgenic mice or *lck* transgenic *RAG-1* mutant mice was equal to or slightly larger than the number in wild-type littermates (Table 1). The previously published numbers of thymocytes in TCRβ transgenic mice or TCRβ transgenic *RAG-1* mutant mice (Mombaerts et al., 1992b) are given for comparison, as well as the number for TCRβ transgenic mice that are homozygous for the severe combined immunodeficiency (*scid*) mutation. These experiments show that an activated *lck* transgene can mimic the action of a TCRβ transgene in the *RAG-1* mutant background, al-

Table 1. Numbers of Total Thymocytes in Crosses between *lck* Transgenic Mice and *RAG-1* Mutant Mice

Line	Type	Number of mice	Average Number of thymocytes as percentage of wild-type (± SD)
pLGF ²⁰⁵⁴	<i>RAG-1</i> ^{-/-}	26	0.95 (± 0.67)
	<i>lck</i> Tg	4	112 (± 22)
	<i>RAG-1</i> ^{-/-} , <i>lck</i> Tg	9	126 (± 41)
pLGF ²⁰⁷³	<i>lck</i> Tg	8	101 (± 28)
	<i>RAG-1</i> ^{-/-} , <i>lck</i> Tg	11	125 (± 31)
pLGF ²⁰⁸²	<i>lck</i> Tg	6	121 (± 44)
	<i>RAG-1</i> ^{-/-} , <i>lck</i> Tg	6	126 (± 22)
pLGC4 ⁷¹²⁰	<i>lck</i> Tg	8	163 (± 29)
	<i>RAG-1</i> ^{-/-} , <i>lck</i> Tg	12	163 (± 48)
TCRβ Tg		17	82 (± 32)
<i>RAG-1</i> ^{-/-} × TCRβ Tg		15	102 (± 31)
<i>scid/scid</i> × TCRβ Tg		6	13 (± 2.4)

WT, wild-type; Tg, transgenic. Mice were analyzed between 19 and 48 days of age. Only litters with at least two wild-type mice were included. The wild-type mice are either *RAG-1*^{-/-} or *RAG-1*^{+/-}. The number of total thymocytes was calculated by counting an aliquot using a hemacytometer, and the numbers for the littermates were converted into a percentage of wild type. The average number of 26 *RAG-1* mutant mice present in the four types of crosses is given at the top. For comparison, the data for *RAG-1*^{-/-} × TCRβ Tg and TCRβ Tg mice are given (taken from Mombaerts et al., 1992b). The numbers for *scid/scid* × TCRβ Tg are new; the same TCRβ transgene was used as for the cross with the *RAG-1* mutant mice.

Table 2. Numbers of Total Thymocytes in Crosses between *lck* Mutant Mice and TCR β Transgenic RAG-1 Mutant Mice

Litter number and age	Mouse number	Type	Number of total thymocytes (in 10 ⁶ cells)
1 34 days	1	WT	140
	2	WT	180
	3	WT	192
	4	TCR β Tg	188
	5	TCR β Tg	232
	6	RAG-1 ^{-/-} , TCR β Tg	228
	7	RAG-1 ^{-/-} , TCR β Tg	280
	8	<i>lck</i> ^{-/-}	3.0
	9	<i>lck</i> ^{-/-} , RAG-1 ^{-/-} , TCR β Tg	3.4
2 19 days	1	WT	108
	2	WT	196
	3	TCR β Tg	200
	4	TCR β Tg	224
	5	TCR β Tg	224
	6	RAG-1 ^{-/-} , TCR β Tg	288
	7	<i>lck</i> ^{-/-}	23
	8	<i>lck</i> ^{-/-} , TCR β Tg	6.0
	9	RAG-1 ^{-/-}	0.4
	10	<i>lck</i> ^{-/-} , RAG-1 ^{-/-} , TCR β Tg	10

Numbers are given for individual mice from two litters. The parents were both RAG-1^{-/-} and *lck*^{-/-}, and one of them was also TCR β transgenic. Any offspring can be heterozygous for either of the two mutations.

though the former seems to cause some "overshooting" in the numbers of DP cells.

TCR β Transgenic *lck* Mutant and RAG-1 Mutant Mice

We next sought to determine whether *lck* is an essential component of the TCR β -mediated transition of DN cells to DP cells, by crossing *lck* mutant mice (Molina et al., 1992) to TCR β transgenic RAG-1 mutant mice. In the latter mice, more than 95% of the thymocytes are DP, and the total number of thymocytes is close to wild-type levels (Table 1) (see also Mombaerts et al., 1992b). When the *lck* mutation was crossed in, the total number of thymocytes was reduced to approximately 5% of wild-type levels. Of these cells, two thirds were DP and they were predominantly small (Table 2; Figure 2). Thus, although the TCR β -mediated DN to DP transition can proceed without the normal function of p56^{lck}, expansion of the DP thymocytes seems to require it.

p56^{lck} May Down-Regulate Surface Pre-TCR Expression

Flow cytometric analysis of TCR β transgenic *lck* mutant and RAG-1 mutant thymocytes uncovered another, as yet undescribed, TCR β -mediated differentiation event in which p56^{lck} appears to play a role.

In TCR β transgenic *scid*, RAG-1, or RAG-2 mutant mice, the expression of the transgenic TCR β and CD3 ϵ chains is not stoichiometric; the former is expressed much more than the latter on the thymocyte surface (Kishi et al., 1991; Mombaerts et al., 1992b; Shinkai et al., 1993). This is in contrast with the TCR-CD3 complexes expressed on the surface of DP or single positive thymocytes in wild-type mice, in which TCR β and CD3 ϵ are stoichiometric. It has been suggested that overexpression of the TCR β on the

thymocytes of these transgenic mice is unphysiological and reflects a transgenic mouse artifact (Groettrup and von Boehmer, 1993a, 1993b). A small fraction of thymocytes in TCR β transgenic RAG-1 mutant mice, however, expresses low levels of TCR β and CD3 ϵ in stoichiometric amounts (see also Mombaerts et al., 1992b): these cells are larger than the bulk of thymocytes in these mice (Figure 3A). Similarly, in TCR α mutant mice, a small fraction of the thymocytes expresses low levels of TCR β and CD3 ϵ on the surface (see also Mombaerts et al., 1992b), and most of these cells are also large (Figure 3B). These TCR β -CD3 ϵ complexes seem to be distinct from the artifactual complexes and could be pre-TCR complexes.

Unlike the thymocytes in TCR β transgenic RAG-1 mutant mice, virtually all thymocytes in TCR β transgenic *lck* mutant and RAG-1 mutant mice expressed CD3 ϵ and TCR β chains in stoichiometric amounts, and at higher levels than TCR α mutant or in TCR β transgenic RAG-1 mutant thymocytes (Figure 2A, bottom). This result suggests that p56^{lck} may also play a role in regulating assembly or expression of a pre-TCR complex.

Discussion

DN to DP Transition Depends on TCR β

The role of TCR β in early thymocyte differentiation was first suggested by the observation that a functionally rearranged TCR β transgene causes an appearance of some DP thymocytes in *scid* mice (Kishi et al., 1991). Since the number of these DP thymocytes was at least an order of magnitude lower than that in the wild-type mice, it was suggested that TCR β is involved only in the differentiation to the DP stage and that expansion of the DP thymocyte pool requires TCR α expression (von Boehmer, 1990). However, this latter hypothesis was challenged by our ob-

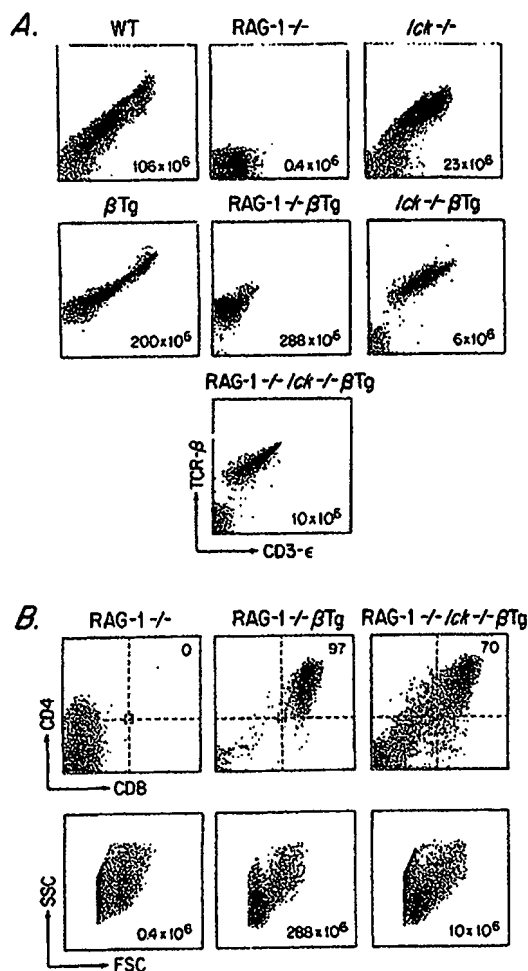


Figure 2. Flow Cytometric Analysis of Thymocytes

(A) Cross between *lck* mutant mice and TCRβ transgenic RAG-1 mutant mice. Staining with CD3e-FITC and TCRβ-PE. These seven mice are 3-week-old littermates, of a cross between a double heterozygous mouse with a double heterozygous, transgenic mouse. WT, wild-type could be heterozygous for either mutation. The total number of thymocytes is indicated in the lower right corner of each panel. In the transgenic double mutant mouse, most thymocytes express CD3e and TCRβ in stoichiometric levels, unlike in the transgenic RAG-1 mutant mouse.

(B) Top, staining with CD4-FITC and CD8-PE. Bottom, forward and side scatter, of selected mice represented in part (A). In the transgenic double mutant mouse, most thymocytes are double positive and small, but their numbers are much reduced compared with the transgenic RAG-1 mutant mouse.

servation that the same functionally rearranged TCRβ transgene can result in an appearance of DP thymocytes in wild-type numbers in the RAG-1 mutant background (Mombaerts et al., 1992b). It was subsequently shown that another TCRβ transgene had the same effect in RAG-2 mutant mice (Shinkai et al., 1993). In the present study, we confirmed that the *scid* background as opposed to the

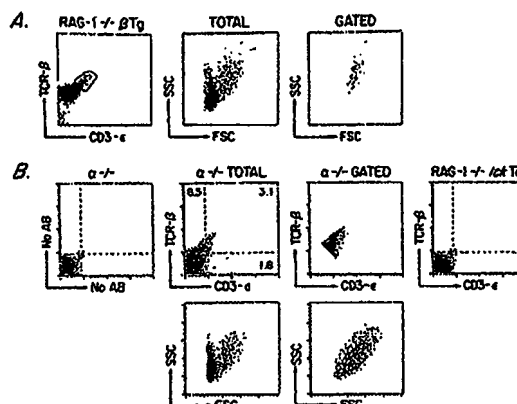


Figure 3. Flow Cytometric Analysis of Thymocytes

(A) TCRβ transgenic RAG-1 mutant mouse. Staining with CD3e-FITC and TCRβ-PE. Forward scatter (FSC) and side scatter (SSC) for total and gated populations are shown. The gated population is indicated in the leftmost panel with lines.

(B) TCRα mutant mouse. (Top) Sequentially shown are the following: sample not subjected to any antibodies and run in parallel; staining with CD3e-FITC and TCRβ-PE, with gates on all thymocytes; staining with CD3e-FITC and TCRβ-PE, with gates only on CD3e-TCRβ-expressing thymocytes. The rightmost panel (*lck* transgenic RAG-1 mutant mouse) is a negative control for the specificity of the antibody staining: these thymocytes are comparable to TCRα mutant thymocytes with regard to size (data not shown), number and expression of CD4 and CD8.

(Bottom) Forward and side scatter of the total and gated TCRα mutant thymocyte population.

RAG-1 mutant background does not allow complete restoration of DP thymocyte numbers by the TCRβ transgene (Table 1). The discrepancy in DP thymocyte numbers between TCRβ transgenic RAG-1 mutant mice and TCRβ transgenic *scid* mutant mice can be explained by pleiotropic effects of the poorly understood *scid* mutation, or lethal aberrant rearrangement events (Bosma and Carroll, 1991). Whereas these experiments showed that a functionally rearranged TCRβ gene can promote early thymocyte development, they did not prove that TCRβ is required for this process. Formal proof of the essential nature of a TCRβ gene in promoting early thymocyte development could only be obtained by analysis of TCRβ mutant mice (Mombaerts et al., 1992b).

Involvement of p56^{lck} in Early Thymocyte Development

The use of genetically manipulated mice has supported the view that the tyrosine kinase p56^{lck} participates in TCRβ-mediated early thymocyte differentiation (reviewed by Owen, 1993; Anderson et al., 1994). The *lck* mutation (10% of wild-type thymocyte numbers) (Molina et al., 1992) blocked thymocyte differentiation at a stage earlier than a double CD4 and CD8 mutation (100% of wild-type numbers) (Schilham et al., 1993). It appeared, therefore, that p56^{lck} functions at an early stage of thymocyte development independent of these coreceptor molecules.

In this paper, we have shown that overexpression of an activated form of $p56^{lck}$, even if it is unable to bind to CD4 and CD8, is able to restore numbers of DP thymocytes to wild-type (or slightly higher) levels in *RAG-1* mutant mice. Thus, overexpression of $p56^{lck}$ seems to be able to deliver the signal for differentiation to the DP stage and subsequent expansion of DP cells that is normally delivered by a V-D-J TCR β chain. However, the phenotype of the TCR β transgenic *lck* mutant and *RAG-1* mutant thymus suggests that $p56^{lck}$ is not required for differentiation to the DP stage. Rather $p56^{lck}$ appears to be required for expansion of DP cells.

These data and their interpretations are consistent with the earlier observation that DP thymocytes appear in small numbers in the *lck* mutant mice (Molina et al., 1992). However, they are not necessarily in line with the previous observations made with dominant negative *lck* transgenic mice. In these mice, the number of DP thymocytes was inversely correlated with the expression level of the transgene, and in the mice expressing the highest levels, no DP thymocyte were detectable (Levin et al., 1993a). As previously noted, the apparent discrepancy may be explained by one of the following several possibilities. First, the *lck* mutation (Molina et al., 1992) may not be a null mutation; truncated protein with some activity may be produced at low levels from the disrupted allele. Second, other kinases such as *Itk/Tsk* (Siliciano et al., 1992; Heyek and Berg, 1993) or ZAP-70 (Chan et al., 1992) may act in pathways parallel to $p56^{lck}$. Functional overlap in the *src* family of tyrosine kinases has recently been proposed for *hck* and *fgr* (Lowell et al., 1994). According to this hypothesis, the failure of the putative parallelly acting kinases to promote the DN to DP transition of thymocytes in the dominant negative *lck* transgenic mice argues that the excess catalytically inactive $p56^{lck}$ sequesters one or more essential components of the signalling pathway that are needed for the functioning of the parallelly acting kinases. Crossing *lck* mutant mice with other targeted mutant mice (Mombaerts, 1993) may reveal the role of such kinases in the DN to DP transition. Third, it is also possible that compensatory signalling pathways emerge in *lck* mutant thymocytes that do not ordinarily act to control thymocyte development. Finally, catalytically inactive $p56^{lck}$ may interfere, when overexpressed at high levels, with the function of other kinases or even of unrelated signalling pathways, perhaps by inhibiting interactions with partners upstream or downstream in the pathway that are shared with other signalling cascades.

Regardless of the precise mechanism involved, $p56^{lck}$ clearly plays a pivotal role in the generation of DP thymocytes in normal numbers.

$p56^{lck}$ May Down-Regulate Surface Pre-TCR Expression

The analysis of the thymocytes from TCR β transgenic rearrangement-deficient mice (*scid*, *RAG-1*, or *RAG-2* mutant mice) with respect to the nature of TCR β containing surface complexes resulted in some confusion. In these mice, many of the transgenic TCR β chains are expressed as monomers, without CD3 ϵ , and in a phosphatidyl inositol-linked form (Groettrup and von Boehmer, 1993a, 1993b).

Such complexes seem to be an artifact of the transgenic mice and have not been observed in immature T cell lines (Punt et al., 1991; Groettrup et al., 1992; Bernard et al., 1993; Mombaerts et al., unpublished data). In this study, a careful analysis of our TCR β transgenic *RAG-1* mutant thymocytes revealed that a minor subset (less than 5%) expressed TCR β and CD3 ϵ in stoichiometric amounts (see also Mombaerts et al., 1992b); these cells differ from the bulk of thymocytes by their larger size. We also showed that a minor (about 5%) thymocyte subset composed of relatively large cells in the TCR α mutant mice, expresses CD3 ϵ and TCR β in stoichiometric amounts. That this low level staining is specific was demonstrated with two appropriate negative controls (see legend to Figure 3B). Others have noticed a similar thymocyte subset in another strain of TCR α mutant mice (Groettrup et al., 1993). Taken together, these data suggest that a small subset of relatively large thymocytes expresses surface complexes that contain TCR β and CD3 ϵ in stoichiometric amounts, but no TCR α . As there is no TCR α antibody useful for flow cytometry, it remains to be seen whether such a subset of cells also exists in wild-type mice.

We observed that the putative pre-TCR is expressed on the surface of virtually all thymocytes in TCR β transgenic *lck* mutant and *RAG-1* mutant mice. The high expression level of the complexes on these thymocytes compared with the thymocytes from TCR α mutant mice can be explained by the presence of the TCR β transgene in multiple copies. Similar surface expression is seen in thymocytes expressing both dominant negative *lck* and TCR β chain transgenes (Anderson et al., 1993). One interpretation of this phenomenon invokes the well-described ability of $p56^{lck}$ to regulate the assembly of TCR-CD3 complexes in DP thymocytes (Nakayama et al., 1993). In this model, $p56^{lck}$ would direct catabolism of CD3 subunits in the endoplasmic reticulum compartment, and thereby down-regulate surface expression of CD3. Thus, the absence of $p56^{lck}$ activity in the TCR β transgenic-*lck* mutant and *RAG-1* mutant thymocytes may lead to up-regulation of the assembly of TCR β -CD3 complexes and hence increased levels of their expression on the cell surface. A second and more provocative possibility is that $p56^{lck}$ acts to block expression of a "chaperone" molecule that accompanies the TCR β chain to the cell surface. This molecule could be, for example, a surrogate TCR α chain that might be coexpressed with TCR β in a pre-TCR complex. A candidate for such a surrogate TCR α chain has recently been described (Groettrup et al., 1993). In the TCR α mutant thymus, signaling through $p56^{lck}$ would lead to down-regulation of surface TCR β expression on the more mature small thymocytes. Likewise, in the TCR β transgenic *RAG-1* mutant thymus, $p56^{lck}$ signaling would also down-regulate pre-TCR surface expression. Consequently, the TCR β chains, which are produced in large amounts from the multiple transgenic copies, would find their way onto the surface of the small thymocytes in a nonphysiological manner. The more profound implication of this hypothesis is that $p56^{lck}$ may normally regulate pre-TCR expression in developing thymocytes.

Experimental Procedures

Mice

RAG-1 mutant mice and TCR β transgenic RAG-1 mutant mice were as described before (Mombaerts et al., 1992a, 1992b). The pLGF transgenic lines were described in Abraham et al., 1992, and the pLGA transgenic line was first reported in Levin et al., 1993b. A description of the phenotype of *lok* mutant mice can be found in Molina et al., 1992. The TCR β transgene was originally described in Uematsu et al., 1988, and mice of line 101 (Krimpenfort et al., 1989) were used.

Flow Cytometry

Flow cytometry was performed as described in detail (Mombaerts et al., 1992b).

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References

- Abraham, K. M., Levin, S. D., Marth, J. D., Forbush, K. A., and Perlmutter, R. M. (1992). Delayed thymocyte development induced by augmented expression of *p56^{lck}*. *J. Exp. Med.* 175, 1421–1432.
- Anderson, S. J., Abraham, K. M., Nakayama, T., Singer, A., and Perlmutter, R. M. (1992). Inhibition of T-cell receptor β -chain gene rearrangement by overexpression of the non-receptor protein tyrosine kinase *p56^{lck}*. *EMBO J.* 11, 4877–4888.
- Anderson, S. J., Levin, S. D., and Perlmutter, R. M. (1993). The protein tyrosine kinase *p56^{lck}* controls allelic exclusion of T-cell receptor β chain genes. *Nature* 365, 552–554.
- Anderson, S. J., Levin, S. D., and Perlmutter, R. M. (1994). Involvement of the protein tyrosine kinase *p56^{lck}* in T cell signaling and thymocyte development. *Adv. Immunol.* 56, 151–178.
- Bernard, O., Groettrup, M., Mugneret, F., Berger, R., and Azogui, O. (1993). Molecular analysis of T-cell receptor transcripts in a human T-cell leukemia bearing a t(1;14) an an inv(7); cell surface expression of a T-cell receptor- β chain in the absence of α chain. *Leukemia* 7, 1645–1653.
- Bosma, M. J., and Carroll, A. M. (1991). The *scid* mouse mutant: definition, characterization and potential uses. *Annu. Rev. Immunol.* 9, 323–350.
- Chan, A. C., Iwashima, M., Turck, C. W., and Weiss, A. (1992). ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell* 71, 649–662.
- Davis, M. M., and Bjorkman, P. (1988). T-cell antigen receptor genes and T-cell recognition. *Nature* 334, 395–402.
- Godfrey, D. I., and Zlotnik, A. (1993). Control points in early T-cell development. *Immunol. Today* 14, 547–553.
- Godfrey, D. I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3⁺CD4⁺CD8⁺ triple-negative adult mouse thymocytes defined by CD44 and CD25. *J. Immunol.* 150, 4244–4252.
- Godfrey, D. I., Kennedy, J., Mombaerts, P., Tonegawa, S., and Zlotnik, A. (1994). Onset of TCR β gene rearrangement and role of TCR β expression during CD3⁺CD4⁺CD8⁺ thymocyte differentiation. *J. Immunol.* 152, 4783–4792.
- Groettrup, M., and von Boehmer, H. (1993a). TCR β chain dimers on immature thymocytes from normal mice. *Eur. J. Immunol.* 23, 1393–1396.
- Groettrup, M., and von Boehmer, H. (1993b). A role for a pre-T cell receptor in T cell development. *Immunol. Today* 14, 610–614.
- Groettrup, M., Baron, A., Griffiths, G., Palacios, R., and von Boehmer, H. (1992). T cell receptor (TCR) β chain homodimers on the surface of immature but not mature α , γ , δ chain deficient T cell lines. *EMBO J.* 11, 2735–2746.
- Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M. J., Hayday, A. C., and von Boehmer, H. (1993). A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor β chain and a 33 kd glycoprotein. *Cell* 75, 283–294.
- Heyek, S. D., and Berg, L. J. (1993). Developmental regulation of a murine T-cell-specific tyrosine kinase gene, *Tsk*. *Proc. Natl. Acad. Sci. USA* 90, 669–673.
- Kishi, H., Borgulya, P., Scott, B., Karjalainen, K., Trautnecker, A., Kaufman, J., and von Boehmer, H. (1991). Surface expression of the β T cell receptor (TCR) chain in the absence of other TCR or CD3 proteins on immature T cells. *EMBO J.* 10, 93–100.
- Krimpenfort, P., Ossendorp, F., Borst, J., Mellef, C., and Berns, A. (1989). T-cell depletion in transgenic mice carrying a mutant gene for TCR β . *Nature* 341, 742–746.
- Levin, S. D., Anderson, S. J., Forbush, K. A., and Perlmutter, R. M. (1993a). A dominant-negative transgene defines a role for *p56^{lck}* in thymopoiesis. *EMBO J.* 12, 1671–1680.
- Levin, S. D., Abraham, K. M., Anderson, S. J., Forbush, K. A., and Perlmutter, R. M. (1993b). The protein tyrosine kinase *p56^{lck}* regulates thymocyte development independently of its interactions with CD4 and CD8 coreceptors. *J. Exp. Med.* 178, 245–256.
- Lowell, C. A., Soriano, P., and Varmus, H. E. (1994). Functional overlap in the *src* gene family; inactivation of *hck* and *lck* impairs natural immunity. *Genes Dev.* 8, 387–398.
- Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K. U., Vellette, A., Davidson, D., and Mak, T. W. (1992). Profound block in thymocyte development in mice lacking *p56^{lck}*. *Nature* 357, 161–164.
- Mombaerts, P. (1993). Dismantling the immune system. *Curr. Opin. Biotech.* 4, 690–698.
- Mombaerts, P., and Tonegawa, S. (1994). Lymphocyte development and function in T-cell receptor and RAG-1 mutant mice. In *Targeted Mutagenesis and Transgenesis in Immunology*, P. Ohashi and H. Bluethmann, eds. (San Diego, Academic Press, Incorporated), pp. 15–34.
- Mombaerts, P., Clarke, A. R., Hooper, M. L., and Tonegawa, S. (1991). Creation of a large genomic deletion at the T-cell antigen receptor β subunit locus in mouse embryonic stem cells by gene targeting. *Proc. Natl. Acad. Sci. USA* 88, 3084–3087.
- Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. E. (1992a). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68, 869–877.
- Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Ithara, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L., and Tonegawa, S. (1992b). Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature* 350, 225–231.
- Nakayama, T., Wiest, D. L., Abraham, K. M., Munitz, T. I., Perlmutter, R. M., and Singer, A. (1993). Decreased signaling competence as a result of receptor overexpression: overexpression of CD4 reduces its ability to activate *p56^{lck}* tyrosine kinase and to regulate T-cell antigen receptor expression in immature CD4⁺CD8⁺ thymocytes. *Proc. Natl. Acad. Sci. USA* 90, 10534–10538.
- Oettinger, M. A., Schatz, D. G., Gorka, C., and Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* 248, 1517–1523.
- Owen, M. J. (1993). T-cell differentiation under control. *Curr. Biol.* 3, 780–782.
- Perlmutter, R. M., Levin, S. D., Appleby, M. W., Anderson, S. J., and Alberola-Ila, J. (1993). Regulation of lymphocyte function by protein phosphorylation. *Annu. Rev. Immunol.* 11, 451–489.
- Philpott, K. L., Viney, J. L., Kay, G., Rastan, S., Gardiner, E. M., Chao, S., Hayday, A. C., and Owen, M. J. (1992). Lymphoid development in mice congenitally lacking T cell receptor $\alpha\beta$ -expressing cells. *Science* 256, 1448–1452.
- Punt, J. A., Kubo, R. T., Saito, T., Finkel, T. H., Kathiresan, S., Blank, K. J., and Hashimoto, Y. (1991). Surface expression of a T cell receptor β (TCR β) chain in the absence of TCR α , δ , and γ proteins. *J. Exp. Med.* 174, 775–783.

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- Schatz, D. G., Oettinger, M. A., and Baltimore, D. (1989). The V(D)J recombination activating gene, *RAG-1*. *Cell* 59, 1035-1048.
- Schliham, M. W., Fung-Leung, W. P., Rahemtulla, A., Krenzig, T., Zhang, L., Potter, J., Miller, R. G., Hengartner, H., and Mak, T. W. (1993). Alloreactive cytotoxic T cells can develop and function in mice lacking both CD4 and CD8. *Eur. J. Immunol.* 23, 1299-1304.
- Shinkai, Y., Rathbun, G., Lam, K.-P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charon, J., Datta, M., Young, F., Stall, A. M., and Alt, F. W. (1992). *RAG-2* deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68, 855-868.
- Shinkai, Y., Koyasu, S., Nakayama, K.-I., Murphy, K. M., Loh, D. Y., Reinherz, E., and Alt, F. W. (1993). Restoration of T cell development in *RAG-2* deficient mice by functional TCR transgenes. *Science* 259, 822-825.
- Siliciano, J. D., Morrow, T. A., and Desiderio, S. V. (1992). *ltk*, a T-cell-specific tyrosine kinase gene inducible by interleukin 2. *Proc. Natl. Acad. Sci. USA* 89, 11194-11198.
- Tonegawa, S. (1983). Somatic generation of antibody diversity. *Nature* 302, 575-581.
- Uematsu, Y., Ryser, S., Dembic, Z., Borgulya, P., Krimpenfort, P., Berns, A., von Boehmer, H., and Steinmetz, M. (1988). In transgenic mice the introduced functional T cell receptor β gene prevents expression of endogenous β genes. *Cell* 52, 831-841.
- von Boehmer, H. (1988). The developmental biology of T lymphocytes. *Annu. Rev. Immunol.* 6, 309-326.
- von Boehmer, H. (1990). Developmental biology of T cells in T-cell receptor transgenic mice. *Annu. Rev. Immunol.* 8, 531-556.